

Novel NMR tools to study structure and dynamics of biomembranes

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Abstract

Nuclear magnetic resonance (NMR) studies on biomembranes have benefited greatly from introduction of magic angle spinning (MAS) NMR techniques. Improvements in MAS probe technology, combined with the higher magnetic field strength of modern instruments, enables almost liquid-like resolution of lipid resonances. The cross-relaxation rates measured by nuclear Overhauser enhancement spectroscopy (NOESY) provide new insights into conformation and dynamics of lipids with atomic-scale resolution. The data reflect the tremendous motional disorder in the lipid matrix. Transfer of magnetization by spin diffusion along the proton network of lipids is of secondary relevance, even at a long NOESY mixing time of 300 ms. MAS experiments with re-coupling of anisotropic interactions, like the ^{13}C – ^1H dipolar couplings, benefit from the excellent resolution of ^{13}C shifts that enables assignment of the couplings to specific carbon atoms. The traditional ^2H NMR experiments on deuterated lipids have higher sensitivity when conducted on oriented samples at higher magnetic field strength. A very large number of NMR parameters from lipid bilayers is now accessible, providing information about conformation and dynamics for every lipid segment. The NMR methods have the sensitivity and resolution to study lipid–protein interaction, lateral lipid organization, and the location of solvents and drugs in the lipid matrix. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Nuclear magnetic resonance (NMR) studies on lipid bilayers have a long history beginning with high-resolution NMR studies on small unilamellar liposomes, continuing with the use of solid-state ^{31}P NMR to study the phase state of the lipid

matrix, and the first precise ^2H NMR order parameter studies on lipid segments in membranes using specifically deuterated phospholipids. More recently attention has been focused on development of magic angle spinning (MAS) NMR techniques.

Over the past decade, the goal of membrane studies has changed. In the early years, emphasis was mostly on the study of biophysical properties of model compounds like 1,2-dipalmitoyl-sn-glycero-3-phosphocholine. More recently, new discov-

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eries in molecular and cell biology have stimulated a wave of biologically-oriented membrane studies with emphasis on properties of complex lipid mixtures that mimic function of specific cell membranes. The influence of the lipid matrix on the function of integral and peripheral membrane proteins is now widely recognized. There is growing appreciation for the important contributions of lipids to lateral organization of membrane constituents, as expressed in the raft hypothesis (Simons and Ikonen, 1997).

In order to meet today's experimental challenges, NMR methods had to evolve. The biggest limitation to application of NMR has been its low sensitivity. Although NMR remains a method that requires significant quantities of membrane material, typical sample size was reduced by one or even two orders of magnitude over the past two decades. While initial order parameter studies were conducted on samples containing of the order of 100 mg of lipid, the use of modern instruments equipped with high-field superconducting magnets has reduced typical sample size to just a few mg. Under favorable conditions, experiments can be conducted even in the μg range. This is the result of several improvements including higher magnetic field strength, better electronics, more efficient probe design, but also of introduction of MAS NMR techniques and widespread use of oriented samples that improve spectral resolution and increase sensitivity.

2. NMR techniques

Membranes are typically studied by solid-state NMR approaches. The resonance signals of lipids in biomembranes are shifted, broadened, and split by anisotropic interactions, like anisotropies of chemical shift, dipole–dipole interactions, and quadrupolar interactions. The lipids in the matrix perform rapid conformational transitions, rotational diffusion, and lateral diffusion. Under conditions of such rapid anisotropic motions, the degree of averaging of anisotropic interactions is an important measure of lipid organization and of lipid conformational freedom (Yeagle, 1993). Spectral resolution in NMR experiments on

biomembranes is achieved either by averaging of anisotropic interactions using fast sample spinning about the magic angle or by taking advantage of the dispersion in effective strength of anisotropic interactions. This paper describes both the more recently introduced MAS NMR approaches and the order parameter studies on reconstituted oriented samples. The goal of this publication is to summarize experience with application of these novel techniques. Examples were selected from the current research program of our laboratory.

2.1. Magic angle spinning (MAS)

In the 1980s several laboratories demonstrated that MAS experiments on membranes result in well resolved NMR spectra of lipids (Herzfeld et al., 1980; Sefcik et al., 1983; Yeagle and Frye, 1987; Oldfield et al., 1987). The debilitating line-broadening of lipid resonances is eliminated by spinning samples at frequencies in the kHz-range around an axis that is tilted at an angle of 54.7° to the magnetic field, a technique called MAS (Mehring, 1983). When the spinning frequency is of the same order of magnitude as the strength of anisotropic interactions expressed in units of Hz, then signal intensity is collected into a center band and weak rotational sidebands. Resolution of centerband resonances is particularly high when the anisotropic interactions have effective axial symmetry and the spin–spin relaxation times are sufficiently long as for resonances in the liquid–crystalline lipid matrix (Davis et al., 1995).

2.1.1. Spectral resolution and sensitivity

The development of MAS rotors that would not leak water when spinning was a non-trivial development essential to the successful application of MAS to lipid membranes, see e.g. (Yeagle and Frye, 1987). For fast spinning, the semi-liquid membrane samples are encapsulated in the spherical volume of Teflon inserts inside the rotors. The material of inserts, rotors, and resonance coils must be magnetic susceptibility-matched to prevent inhomogeneous broadening of resonance lines from magnetic field gradients caused by the equipment. The MAS technique reduces the

linewidth of lipid proton resonances from several kHz to values in the range of 3–20 Hz, even at modest MAS spinning frequencies in the low kHz-range (Holte and Gawrisch, 1997). This is close to the linewidth of molecules in solvents as studied in high-resolution NMR experiments. The resolution achieved by MAS techniques is equivalent or better than resolution of resonances from very small unilamellar liposomes with diameters below 100 nm that tumble rapidly enough to eliminate anisotropic interactions (Lichtenberg et al., 1975). However, in contrast to liposome studies, for MAS NMR the membranes are not required to have small radii of curvature achieved by intrusive sample preparation procedures like sonication, and water content does not matter as long as the lipids remain in the liquid–crystalline state.

The small linewidth of resonances requires a very stable magnetic field. This is of particular importance when data are acquired over long periods of time, e.g. in multidimensional experiments. For longer acquisitions the use of an internal deuterium lock is strongly advised. The lock-signal is conveniently generated by using double or triple resonance MAS probes that include a deuterium channel. The signal of a few microliters of deuterated water in spinning membrane samples is well resolved and has sufficient strength for locking the field.

Proton resonance experiments just micro- to mg of sample for sufficient signal-to-noise ratios. The ^{13}C NMR experiments require a few mg of membrane material at natural abundance of ^{13}C and up to two orders of magnitude less with isotope enrichment. Experiments can be conducted on

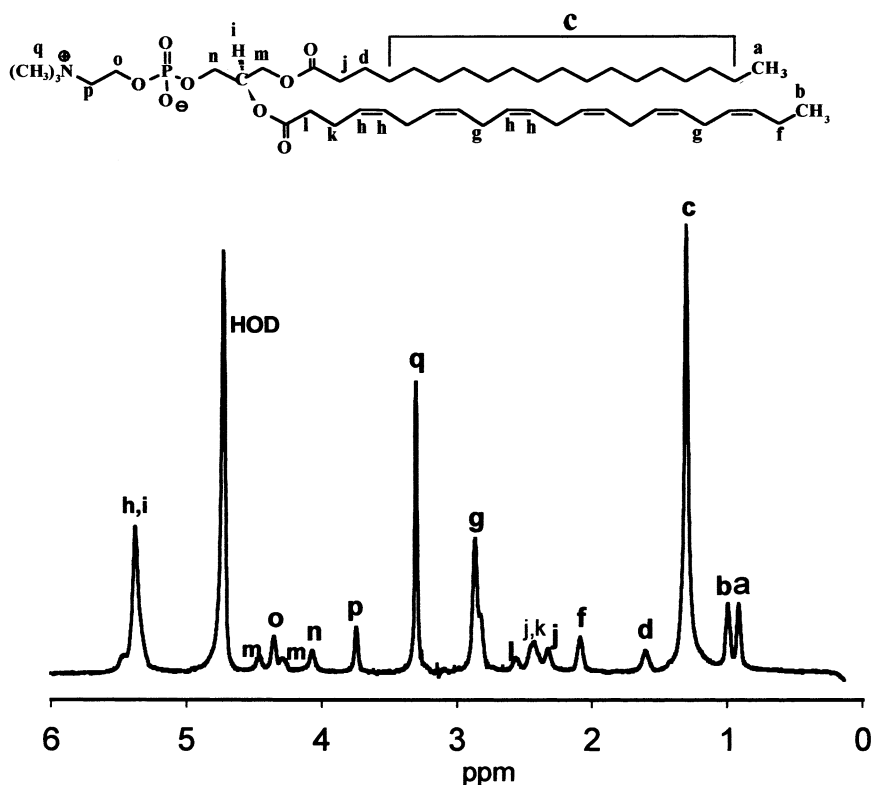


Fig. 1. ^1H MAS NMR spectrum of mixed-chain 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine (18:0–22:6n3 PC) bilayers in 50 wt.% D_2O recorded at a resonance frequency of 500.1 MHz, a temperature of 25 °C, and a MAS spinning speed of 10 kHz. Proton resonance signals of lipids in bilayers have a typical width at half height of 3–20 Hz.

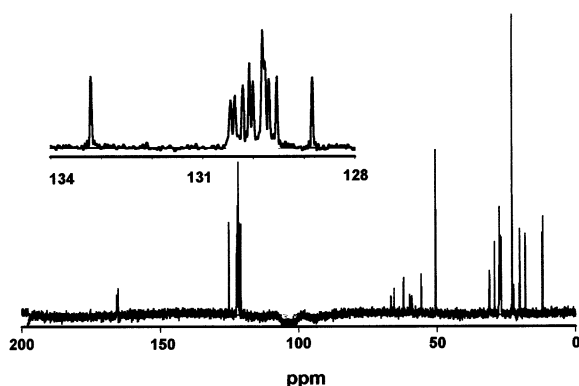


Fig. 2. ^{13}C MAS NMR spectrum of mixed-chain 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine (18:0–22:6n3 PC) bilayers in 50 wt.% D_2O recorded at a resonance frequency of 125.7 MHz, a temperature of 25 °C, and a MAS spinning speed of 10 kHz. Spectra were recorded with 50 kHz of proton-noise decoupling turned on during acquisition of the free induction decay. Signal intensity was enhanced by the nuclear Overhauser effect, achieved by weak decoupling (2 kHz) starting 3 s before detection of the free induction decay. The ^{13}C resonance signals have a linewidth of 5–20 Hz. The excellent resolution is demonstrated for the resonance signals of double bonds in the polyunsaturated sn-2 chain (insert). Eleven resonance signals from the 12 carbons in double bonds are resolved.

model membranes, cells, and even tissues with almost the same degree of resolution (see Figs. 1–3). Resonances of membrane lipids as well as of small membrane-incorporated molecules can be resolved.

2.1.2. Selection of optimal spinning speed

Although reasonable resolution of lipid resonances in the spinning centerband is adequately achieved at spinning frequencies of a few kHz, the use of higher spinning frequencies is recommended. Best performance for most experiments is achieved at spinning frequencies of 10 kHz or higher (Huster et al., 1999). At such frequencies the intensity of spinning sidebands is negligible for all lipid signals, therefore, simplifying data analysis. At lower spinning frequencies the resonances of lipid segments with higher order parameters, e.g. the resonances of lipid glycerol and upper hydrocarbon chain regions, have noticeable intensity in rotational sidebands that re-

sult in selective reduction of these signal intensities in the centerband.

Although high spinning frequencies are desirable, faster spinning has its drawbacks. At spinning frequencies up to 5 kHz, the sample temperature is typically a few degrees lower than the temperature of the compressed gas that drives the rotor. This is the result of the Joule–Thomson effect in the expanding gas. At higher spinning frequencies, the friction between the bearing gas and the surface of the spinning rotor, as well as the induction of eddy currents in conductive samples, result in heating. While the frictional sample heating is predictable, the heating from eddy currents in conductive samples is harder to forecast. In experiments on low-conductivity lipid water dispersions, at a spinning frequency of 10 kHz, the temperature increases by 5–10 °C inside the 4 mm rotors. Higher spinning frequencies result in exponential increase of sample temperature that necessitates the use of very cold gas for the rotor bearings. The sample temperature inside the spinning rotor is conveniently calibrated by recording known phase transition temperatures of

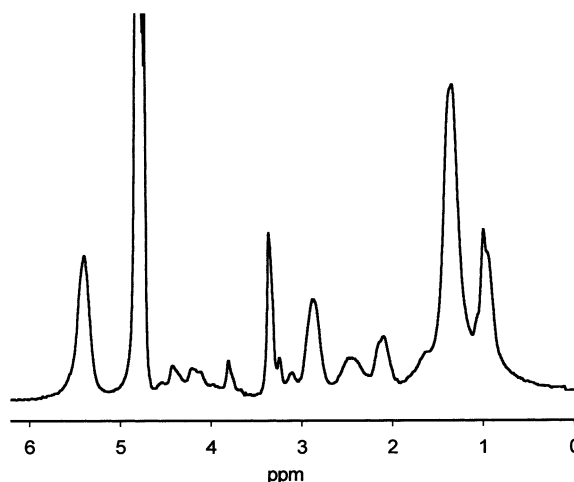


Fig. 3. ^1H NMR spectrum of intact rod outer segment disk membranes from bovine retina in D_2O recorded at a resonance frequency of 500.1 MHz, a temperature of 25 °C, and a MAS spinning speed of 10 kHz. Although spectral resolution is reduced compared with experiments on pure lipid membranes (compare with Fig. 1), all proton resonances of lipids are still well resolved. Resonance signals of membrane proteins (primarily rhodopsin) are broadened beyond detection.

lipids. Since the rotors heat primarily from the gas bearings at both ends, the temperature measurement must be conducted after sufficient equilibration time. Large samples may experience significant temperature gradients seen as a broadening of phase transitions.

The strong centrifugal forces inside a spinning rotor are also of concern. Depending on the spinning frequency, the sample dimensions, and the density differences between membrane material and water, these forces either concentrate the membranes at the inner lining of the rotor wall (H_2O) or in the center of the spinning rotor (D_2O). It was shown (Fritzhanns et al., 1997; Nagle et al., 1999) that centrifugal forces result in modest membrane dehydration that can be safely ignored for most applications.

2.2. Nuclear Overhauser enhancement spectroscopy (NOESY)

The excellent resolution of lipid resonances allows application of techniques that probe magnetization transfer between protons, like nuclear Overhauser enhancement spectroscopy (NOESY), well known for its important contribution to structural determination of soluble proteins (Macura et al., 1981; Ernst et al., 1991). Although a significant number of papers on such bilayer experiments have been published in the past 15 years (Forbes et al., 1988a,b; Ellena et al., 1985; Xu and Cafiso, 1986; Halladay et al., 1990; Chen et al., 1996; Chen and Stark, 1996; Zhou et al., 1997; Gabriel and Roberts, 1987; Volke and Pampel, 1995; Zhou et al., 1999), interest in NOESY experiments was limited because, until recently, cross-relaxation data of lipid bilayers could not be interpreted quantitatively.

We converted NOESY into a tool for quantitative membrane structural studies. The theoretical foundation of our approach was published in the years 1999–2000 as a series of publications in the *Journal of the American Chemical Society* (Huster and Gawrisch, 1999; Feller et al., 1999; Yau and Gawrisch, 2000) and in the *Journal of Physical Chemistry* (Huster et al., 1999).

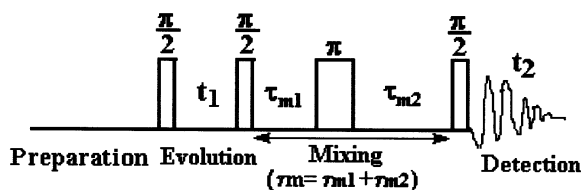


Fig. 4. Pulse sequence for measuring NOESY cross-relaxation rates in bilayers. For suppression of crosspeaks from J -couplings between protons an additional π -pulse at a random position during the mixing time τ was inserted (Ernst et al., 1991).

2.2.1. Pulse sequence

Proton magnetization is flipped into the x – y plane of the rotating coordinate system by a 90° pulse (see Fig. 4). During the incremented delay time, t_1 , the proton magnetization spreads in the x – y plane according to the differences in chemical shift between resonance signals and to the scalar couplings between protons. After the time period, t_1 , the magnetization is flipped back to the z -axis. The effective intensity of magnetization vectors along z is a function of the phase of magnetization relative to the phase of the radio frequency B_1 -field. During the mixing time, τ , when magnetization is along the z -axis, nuclei that are coupled by dipolar interaction exchange magnetization, resulting in correlated intensity changes of signals from interacting nuclei. At the end of the mixing time the magnetization is flipped back to the x – y plane by another 90° pulse, and the resulting free induction decay is acquired (time domain t_2) Fig. 5.

It is recommended to record the two-dimensional (2-D) NOESY spectra in the phase sensitive mode. Spectra of the lipid matrix are acquired typically with 1024–2048 data points in time domain t_2 and 128–512 time increments in time domain t_1 . Depending on signal intensity, from 8 to 64 scans per t_1 time increment are acquired. Mixing times for membrane applications cover the range from 1 ms to 1 s. Most crosspeaks have highest intensity between 300 and 600 ms (Huster et al., 1999; Huster and Gawrisch, 1999). The delay time between scans, d_1 , must be at least three times the longest spin-lattice relaxation time of protons in the membrane, that is $d_1 = 6$ –12 s.

2.2.2. Spectral artifacts

The performance of the 2-D NOESY experiment depends mostly on residual t_1 -noise in the spectra that results from temperature instabilities and from mechanical vibrations in the probe related to sample spinning. Temperature instabilities are easily controlled by supplying temperature-controlled gas to the MAS probe and by controlling gas temperature with a feedback from a sensor inside the probe. The mechanical vibrations are more difficult to control. Most likely they arise from vibrations of the radio frequency coil. The use of well-balanced rotors and of sturdier coil materials improves performance.

Signal intensity may be perturbed by superposition with spinning sidebands that have been folded

back on the centerband and by the presence of J-crosspeaks due to the presence of single-quantum or double quantum coherences during the mixing time (COSY peaks). The interference from sidebands in the f_2 -frequency domain is easily removed by filtering and/or higher spinning frequencies. Filtering is not an option for elimination of sidebands in the f_1 -frequency domain, but interference from superposition with sidebands can be eliminated by choosing the appropriate spinning frequency and/or carrier frequency. Pampel and Volke suggested conditions to superimpose center- and sideband intensities to avoid perturbation (Pampel and Volke, 1997).

We conducted experiments with rotor-synchronized radio-frequency pulses to see if the NOESY

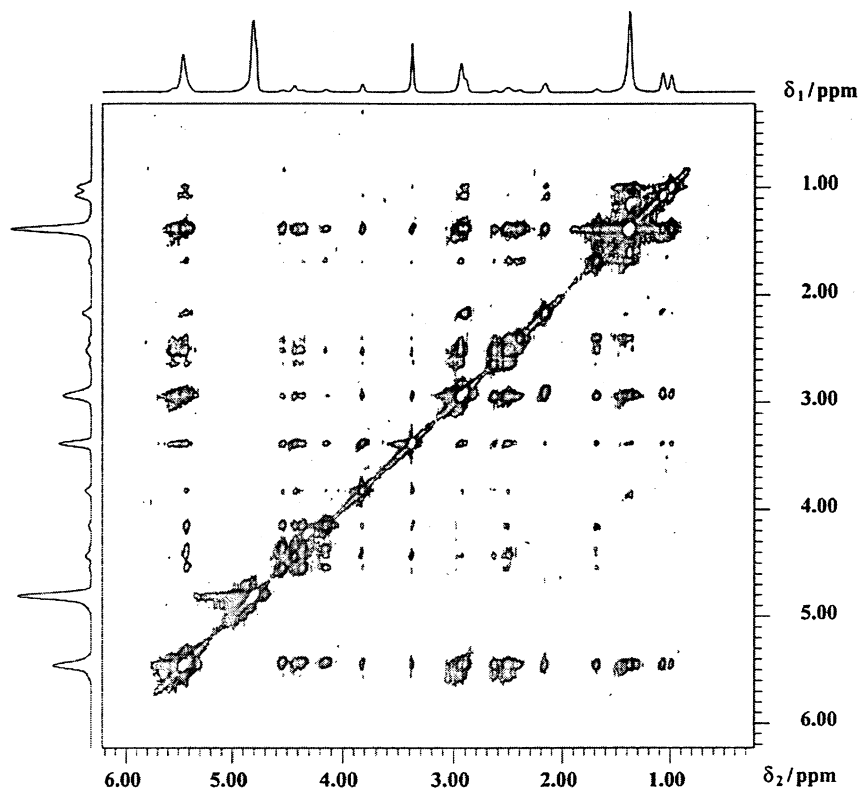


Fig. 5. Two-dimensional NOESY MAS ^1H NMR spectrum of mixed-chain 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine (18:0–22:6n3 PC) bilayers in 50 wt.% D_2O recorded at a mixing time of 300 ms. The diagonal peaks correspond to the 1-D spectrum shown in Fig. 1. The off-diagonal peaks indicate magnetization transfer between the protons. Even distant protons like the choline resonances of the headgroup at 3.3 ppm and the terminal methyl group resonances of hydrocarbon chains at 0.99 ppm transfer magnetization. In experiments on specifically deuterated lipids we demonstrated that such transfers occur between neighbored lipid molecules (Huster and Gawrisch, 1999). They are a reflection of disorder in the lipid matrix.

sequence interferes with rotational echos. At spinning frequencies of 5 kHz and higher we did not observe any influence from rotor synchronization on results because sidebands have only a few percent of the intensity of centerband resonances.

The contributions from J-crosspeaks are easily identified by their oscillation as a function of mixing time τ . We observed J-crosspeaks between glycerol resonances, but also between the chain- and the headgroup resonances, in particular for experiments on polyunsaturated lipid bilayers. The oscillating behavior of contributions from J-crosspeaks provides efficient means to scramble them. We refrained from recording data with variable mixing time as suggested in the literature, because this interferes with the precision of crosspeak analysis. Instead we used a pulse sequence with an additional π -pulse located at random within the mixing time period (Macura et al., 1981). The position of the π -pulse is shifted with every t_1 -increment. This additional pulse has no influence on magnetization exchanged by the Overhauser effect but alters the phase of J-crosspeaks at random. The degree of averaging of these peaks depends on the strength of the J-coupling and the length of the mixing time. Stronger couplings and longer mixing times favor elimination. There is an insignificant contribution to t_1 -noise from scrambling of J-crosspeaks.

2.2.3. Data analysis

The rate of magnetization transfer between resonances is reflected by the intensity of the crosspeaks located off the diagonal. As a function of mixing time, the crosspeaks initially increase in intensity and then at long mixing times, decrease in intensity (see Fig. 6). The slope of the increase at short mixing times reflects the cross-relaxation rate while the loss of magnetization at long mixing times is the result of spin-lattice relaxation. The intensity of diagonal peaks in the spectrum shown in Fig. 5. decreases with increasing mixing time due to magnetization loss by spin-lattice relaxation and to magnetization exchange between resonances.

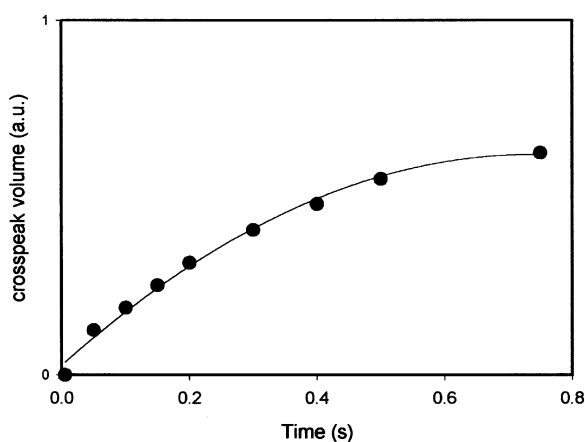


Fig. 6. Intensity of the crosspeak between the terminal methyl group of docosahexaenoic acid and the methyl groups of choline in 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine (18:0–22:6n3 PC) bilayers as a function of mixing time. At short mixing times crosspeak intensity increases linearly with mixing time. At longer mixing times the loss of magnetization due to spin-lattice relaxation becomes apparent. In lipid bilayers the highest crosspeak intensities are observed for mixing times in the range from 300 to 600 ms.

The time dependence of diagonal- and crosspeaks is described by first-order rate kinetics (Macura et al., 1981; Perrin and Gipe, 1984; Bremer et al., 1984; Huster et al., 1999). The relaxation rate matrix (\mathbf{R}) is linked to the crosspeak integral matrix, $A(\tau)$, at a mixing time τ by the equation, $A(\tau) = \exp(-\mathbf{R}\tau)A(0)$, where, $A(0)$ is the peak integral matrix at a mixing time zero. The matrix, \mathbf{R} , describes the loss of magnetization of diagonal peaks to spin-lattice relaxation and the magnetization exchange between all resonances by cross-relaxation

$$\mathbf{R} = \begin{bmatrix} \rho_{11} & \sigma_{12} & \sigma_{13} & \sigma_{14} & \dots & \sigma_{1N} \\ \sigma_{21} & \rho_{22} & \sigma_{23} & \sigma_{24} & \dots & \sigma_{2N} \\ \sigma_{31} & \sigma_{32} & \rho_{33} & \sigma_{34} & \dots & \sigma_{3N} \\ \sigma_{41} & \sigma_{42} & \sigma_{43} & \rho_{44} & \dots & \sigma_{4N} \\ \dots & \dots & \dots & \dots & \dots & \dots \\ \sigma_{N1} & \sigma_{N2} & \sigma_{N3} & \sigma_{N4} & \dots & \rho_{NN} \end{bmatrix}$$

where the σ_{ij} represent cross-relaxation rates and the ρ_{ii} effective relaxation rates of the diagonal peaks. According to the convention, σ_{ij} is the

cross-relaxation rate of magnetization transfer from spin j to spin i , and σ_{ji} of transfer from spin i to spin j . The effective relaxation rate is $\rho_{ii} = R_{ii} - \Sigma \sigma_{ji}$ ($j = 1, \dots, N$), where R_{ii} is the spin lattice relaxation rate of the diagonal peaks. The relaxation rate matrix is not symmetrical if the number of magnetically equivalent spins per resonance varies. However, there is symmetry in the per-proton transfer rates, and the cross-relaxation rates satisfy the symmetry condition $n_j \sigma_{ij} = n_i \sigma_{ji}$, where n_j and n_i are the number of spins in resonances j and i , respectively. The relaxation rate matrix, \mathbf{R} , is calculated by rewriting equation $A(\tau) = \exp(-R\tau)A(0)$, in terms of the matrix of eigenvectors, \mathbf{X} , and the diagonal matrix of eigenvalues, \mathbf{D} , of the normalized peak volume matrix $\mathbf{a}(\tau) = \mathbf{A}(\tau)(\mathbf{A}(0))^{-1}$.

The diagonal- and crosspeak peak volumes are obtained from the 2-D NOESY spectra by integration. Superimposed resonance signals in the 2-D spectra were deconvoluted by fitting Gaussian functions to the peaks. We noticed that diagonal peak intensity equivalent to zero mixing time has better reproducibility when recorded at finite mixing times in the range from 1 to 5 ms. For data analysis it is sufficient to record crosspeak intensity at zero mixing time and at one non-zero mixing time. However, precision can be greatly enhanced by recording peak intensities at several mixing times. The data points recorded at shorter mixing times are particularly valuable, provided that the signal-to-noise ratio is acceptable.

2.2.4. Interpretation of cross-relaxation rates

The NOESY cross-relaxation rates depend on the motions of molecular vectors connecting the interacting protons as expressed through the magnetic dipolar interaction correlation function

$$C_{ij}(t) = \frac{4}{5} \sum_i \sum_j \left\langle \frac{Y_{20}(\vec{r}_{ij}(0))}{r_{ij}^3(0)} \frac{Y_{20}(\vec{r}_{ij}(t))}{r_{ij}^3(t)} \right\rangle$$

where $Y_{20} = [5/16\pi]^{1/2}(3\cos^2\theta - 1)$, and θ is the angle between the internuclear vector, \vec{r}_{ij} , and the normal to the membrane (Brüschweiler and Wright, 1994). The summations over i and j include all magnetically equivalent protons of each resonance. The fluctuating magnetic interactions yield a spectral density that is given by the

Fourier transform of the sum of auto-correlation functions for the magnetic dipole–dipole interactions between all spins of resonances i and j , $J_{ij}(\omega) = \int_{-\infty}^{\infty} C_{ij}(t) \cos(\omega t) dt$. Cross-relaxation rates depend on $J_{ij}(2\omega_0)$ and $J_{ij}(0)$ according to $\Gamma_{ij} = \zeta [3J_{ij}(2\omega_0) - 1/2J_{ij}(0)]$, where, ω_0 is the proton Larmor frequency, and $\zeta = (2\pi/5)\gamma^4\hbar^2(\mu_0/4\pi)^2$, where γ is the gyromagnetic ratio for protons.

When interpreting the Γ_{ij} it is important to emphasize that these cross-relaxation rates are determined both by the probability of close contact between protons and by changes in length and orientation of the vector connecting the interacting protons. The probability of close contact is measured by the magnitude of $C_{ij}(0)$ which is proportional to $\langle 1/r_{ij}^6 \rangle$ and thus extremely sensitive to the number of very close contacts. The dependence of Γ_{ij} on changes in vector length and orientation is seen most easily by considering a simple single exponential decay of the correlation function:

$$\begin{aligned} C(t) &= C(0)e^{-t/\tau} \rightarrow J(\omega) = \frac{2C(0)\tau}{1 + \tau^2\omega^2} \rightarrow \Gamma \\ &= C(0)\zeta \left[\frac{6\tau}{1 + 4\tau^2\omega_0^2} - \tau \right] \end{aligned}$$

Due to the difference in sign between the contributions of $J_{ij}(2\omega_0)$ and $J_{ij}(0)$ to Γ_{ij} , correlation times, τ , longer than ~ 400 ps lead to negative cross-relaxation rates for a proton Larmor frequency of 500 MHz.

There are important differences between cross-relaxation in the more rigid proteins and the very flexible lipids in the matrix of biomembranes. While in proteins the distances between protons is constant and the correlation function of dipole–dipole interaction decays as a result of reorientation of the vector connecting the protons, in liquid–crystalline lipid bilayers the distances between lipid protons is variable, and the correlation function decays as a result of both distance variation and reorientation. The surprising observation has been, that in the lipid matrix the magnetization is transferred between all lipid resonances, but at different rates. Even the most distant protons, like methyl groups of the choline

headgroup and methyl groups at the end of lipid hydrocarbon chains exchange magnetization. Before our papers were published, this observation was explained by a process called spin diffusion in which magnetization between protons is transferred indirectly as a multiple-step process. However, our experiments on protonated lipid in a deuterated matrix (Huster et al., 1999) and on binary mixtures of specifically deuterated lipids (Huster and Gawrisch, 1999) demonstrated unambiguously that spin diffusion is insignificant for most resonances at mixing times up to 300 ms. The direct intermolecular transfer of magnetization between lipid protons is much more efficient. Therefore, the multitude of crosspeaks is a reflection of molecular disorder in the lipid matrix of biomembranes.

A deeper interpretation of cross-relaxation rates is feasible when experimental results are compared with calculated rates from molecular dynamics simulations on a model of the investigated bilayer (Feller et al., 1999). The correlation functions of proton dipole–dipole interactions are conveniently calculated from the trajectories of lipid movement in the bilayers. A time resolution of trajectories of the order of 1 ps is sufficient. The length of the calculation should be such that lateral diffusion of lipids in the plane of the bilayer is covered, equivalent to simulation times of 10 ns or longer. In first approximation, comparison of experiments and simulations revealed that ^1H NMR NOESY cross-relaxation rates report the statistics of contacts between lipid segments of neighboring lipid molecules. Highest rates are measured between protons that have a large overlap in their spatial distribution functions along the bilayer normal, e.g. the protons in the glycerol and the upper hydrocarbon chain segments. Much lower rates are measured for contacts between the lipid headgroup and the hydrocarbon chains that have less overlap of their distribution functions (Huster et al., 1999; Huster and Gawrisch, 1999; Feller et al., 1999). There is a secondary dependence of cross-relaxation rates on differences in correlation times and differences in proton–proton distances of closest approach (Feller et al., 2002).

Membranes are 2-D fluids with very little similarity to rigid solid structures. The lipids in bilayers are very disordered. The position of lipid segments in bilayers is a wide distribution function. Chain tilt, chain upturns, and lipid headgroup movement towards the hydrocarbon phase enable some magnetization exchange between hydrophilic and hydrophobic membrane regions. Although we did not obtain evidence for contact between choline headgroups and methyl groups at the ends of hydrocarbon chains within the same lipid, there are such interactions between neighboring lipid molecules (Huster and Gawrisch, 1999).

The fitting of the autocorrelation functions from the simulation by a sum of exponentially decaying functions yields characteristic correlation times and weight factors determining the relative contribution of the individual motions to cross-relaxation. In experiments on specifically deuterated lipids we established that almost all magnetization transfer occurs between protons of neighboring lipid molecules and scales by a single relaxation mechanism that has the correlation time and activation energy of lateral lipid diffusion (Yau and Gawrisch, 2000). The faster motions have impact on the cross-relaxation rates by influencing the amplitude factor $C(0)$ that is paired with the correlation time of slowest motion.

Measurement of ^1H MAS NOESY cross-relaxation rates permits the study of lipid structure and dynamics as well as of the lateral organization in mixed lipid bilayers. Furthermore, we located successfully small molecules, such as ethanol, and indole analogs inside the lipid matrix with atomic resolution (Holte and Gawrisch, 1997; Yau et al., 1998; Feller et al., 2002).

2.3. Dipolar recoupling on-axis with scaling and shape preservation (DROSS)

While MAS experiments enable almost liquid-like resolution of resonance signals, they also average anisotropic interactions that report about conformation and dynamics of lipids in the matrix of bilayers. It was shown that the anisotropic interactions are re-coupled by application of a

series of radio-frequency pulses that are transmitted with synchronization to the phase of the spinning rotor (Tycko et al., 1989). Two-dimensional experiments can be designed such that along one spectral dimension the superior resolution of resonance signals is maintained while in the other dimension the anisotropic interactions are re-coupled. In the 2-D DROSS experiment (Gross et al., 1997), the f_2 -frequency domain reports the ^{13}C chemical shift while the f_1 -domain reports the ^{13}C – ^1H -dipolar interactions. The experiment benefits from the better resolution of ^{13}C chemical shifts compared with protons. For a typical lipid, more than 20 dipolar couplings can be measured with assignment to specific carbon atoms.

2.3.1. Pulse sequence

The proton magnetization is flipped into the x – y plane of the rotating coordinate system by a 90° pulse (see Fig. 7.). It evolves in the x – y plane under the influence of proton chemical shifts and the dipolar interactions with nearby ^{13}C nuclei. The averaging of ^{13}C – ^1H -dipolar interactions by MAS is prevented by rotor-synchronized pulses on the ^{13}C -channel (Tycko et al., 1989). The

simultaneous π -pulses on the ^1H - and ^{13}C channels, applied at the center of the evolution period, ensure that the chemical shifts are refocused at the time point t_1 . In the second part of the experiment, the proton magnetization is transferred via the scalar ^{13}C – ^1H -coupling to the directly bonded ^{13}C nuclei by a refocused Insensitive Nucleus Enhancement by Polarization Transfer (INEPT) sequence. Magnetization is detected on the ^{13}C channel under strong proton-noise decoupling. Experiments are typically conducted with acquisition of 4K data points in the t_2 -time domain and 32 data points in the t_1 -time domain. The number of scans acquired per t_1 -increment depends on the sample size, typically 64–256 scans for 10–20 mg of lipid on our instrument. The delay time between scans should be at least three times the longest proton spin-lattice relaxation time.

2.3.2. Spectral artifacts

The experiment requires very stable sample spinning achieved by an optical feedback from the spinning rotor to the MAS pneumatic control unit. This control signal is also routed to the pulse programmer of the NMR instrument to synchronize the radio frequency pulses with the phase of the spinning rotor. Due to the finite length of the π -pulses on the ^{13}C channel, the spinning frequencies are typically restricted to values of less than 7 kHz. The rotor synchronization requirements determine the length of the t_1 -time increments that must be multiples of the rotor period, reducing the spectral width in the f_2 -domain to values of 7 kHz or less. This is acceptable for experiments on lipids, since the spectral width of the Pake-doublts representing ^{13}C – ^1H -interactions are reduced by the re-coupling sequence, e.g. a factor of 0.393 for the 4π pulse recoupling scheme (Tycko et al., 1989). The experiment requires very precise adjustment of the π -pulse length on the carbon channel. Any residual magnetization in the x – y plane from the train of π -pulses that is transmitted on the ^{13}C channel for re-coupling of dipolar interactions translates into signals that distort the ^{13}C -free induction decay. Proper phase cycling of the ^{13}C - π -pulses reduces spectral artifacts.

The broad dipolar-split resonance signals in the f_1 -domain decay rather rapidly, reducing the

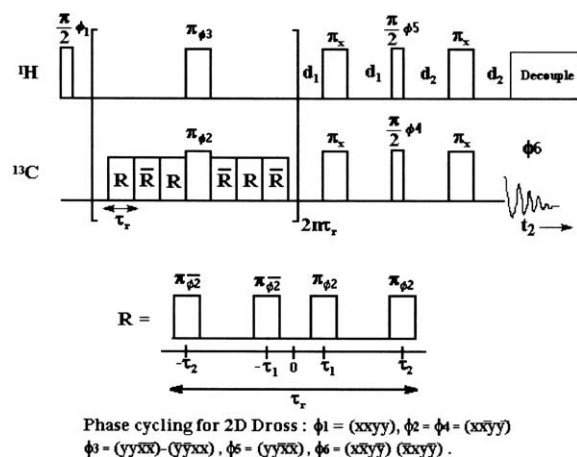


Fig. 7. Pulse sequence for the DROSS experiment (Gross et al., 1997). The magnetic ^{13}C – ^1H -dipolar interaction is re-coupled by a series of four π -pulses per revolution of the rotor (Tycko et al., 1989). The radio-frequency pulses are synchronized to the phase of the spinning rotor by triggering the pulse programmer of the NMR instrument to an optical signal from the spinning rotor.

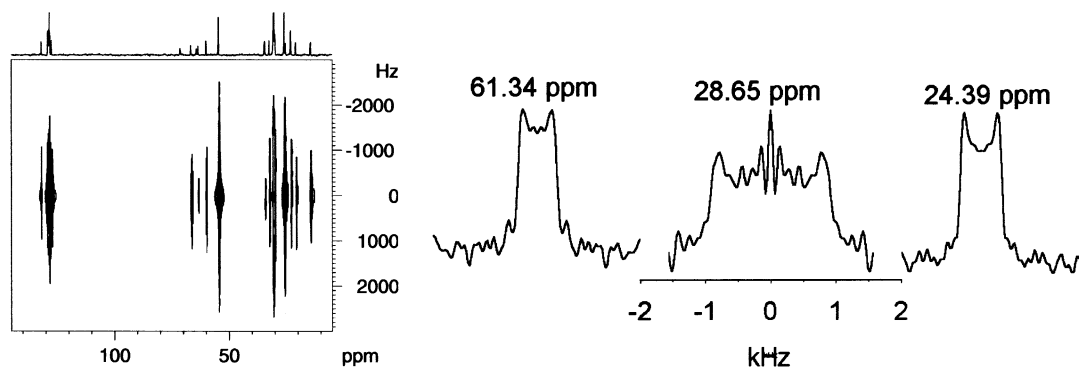


Fig. 8. Two-dimensional DROSS spectrum of 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine (18:0–22:6n3 PC) bilayers in 50 wt.% D₂O recorded at a MAS spinning speed of 6 kHz. Columns from Dross spectra corresponding to hydrocarbon chain (2.39 ppm, 28.65 ppm) and headgroup signals (61.34 ppm) are shown on the right. The Pake doublets are the result of re-coupling of dipolar interactions between the ¹H and ¹³C nuclei. The value of order parameters of ¹³C–¹H bonds is directly proportional to the magnitude of splittings.

range of useful t_1 -time increments to 32. For purposes of processing, the t_1 -dimension is zero-filled to 128 data points. Nevertheless, the severe restriction in size of the t_1 -domain reduces spectral resolution and may result in artifacts. Careful adjustment of spectral base-planes before Fourier transformation in the f_1 -domain is required. The choice of apodization functions for the f_1 -domain is particularly critical. We obtained best results with exponential functions equivalent to a line broadening of 100–200 Hz.

2.3.3. Interpretation of results

The ¹³C–¹H-dipolar interaction reports the orientation of C–H bonds with respect to the bilayer normal and is, therefore, equivalent to C–²H bond order parameters measured by ²H NMR. However, the value for the rigid lattice dipolar C–H coupling is only 20.2 kHz (Gross et al., 1997), compared with a value of $3/4e^2qQ/h = 125$ kHz for ²H-quadrupole splitting in C–²H bonds (Burnett and Muller, 1971). As a consequence, the fast-exchange limit for ²H NMR is of the order of 10^{-5} s, compared with 10^{-4} s for the DROSS experiment. Lipid dynamics with correlation times that are shorter than the fast exchange limit result in averaging of anisotropic interactions. If lipids move with significant spectral density in the range from 10 to 100 kHz, then order parameters detected by the DROSS experiment may be lower

than ²H NMR order parameters. This is the frequency range of undulatory motions of lipid bilayers (Bloom and Evans, 1991). In measurements on multilamellar liposomes no differences between order parameters detected by the DROSS- and ²H NMR experiments have been found (Gross et al., 1997). This indicates that the influence of such undulatory motions is negligible.

The DROSS experiment enables assignment of order parameters to specific carbon atoms (see Fig. 8). Considering the good resolution of ¹³C-resonances of lipid bilayers, this enables order parameter measurements for many lipid segments without the need for specific labeling, a tremendous saving of resources. Unfortunately, this advantage is paired with limited spectral resolution in the f_1 -spectral domain that reports the strength of ¹³C–¹H-dipolar interaction. The ²H NMR experiments on lipids with perdeuterated hydrocarbon chains are complementary to the DROSS experiment because the saturated chains have significant ¹³C-signal superposition that limits the measurement of assigned order parameters. The DROSS experiment can be conveniently applied to the study of polyunsaturated hydrocarbon chains that have better resolution of ¹³C-chemical shifts. Furthermore, the experiment offers a convenient approach for determination of the sign of order parameters (Gross et al., 1997). In contrast, the determination of the sign of ²H NMR order

parameters is less direct and more cumbersome (Morrison and Bloom, 1994).

2.4. Oriented membranes

When experiments are conducted on membranes without MAS, then NMR resonance peaks are split and shifted by anisotropies of chemical shift, dipolar interactions, and quadrupolar interactions. The magnitude of such shifts and splittings depends on the orientation of the bilayer normal with respect to the magnetic field. In samples of unoriented membranes, the signals are all superimposed, resulting in poorly resolved resonance spectra of low intensity. Resonance lines become narrow and intense again when bilayers are well oriented. A single resonance line is measured for signals that have an axially symmetric anisotropy of chemical shift like the ^{31}P NMR signal of lipids in membranes (Seelig, 1978). The chemical shift of this signal is $\nu = \nu_{\text{iso}} + 1/3\Delta\nu_{\text{eff}}\langle 3\cos^2(\theta - 1) \rangle$, where ν_{iso} is the resonance frequency of molecules that perform isotropic reorientation, $\Delta\nu_{\text{eff}}$ is the effective anisotropy of chemical shift, and θ is the angle between the bilayer normal and the magnetic field. The signals of ^2H -labeled groups in lipids become well-resolved doublets with a splitting of $\Delta\nu = 3/4e^2qQ/h\langle 3\cos^2(\theta - 1) \rangle$, where $3/4e^2qQ/h = 125$ kHz (Seelig, 1977). The brackets symbolize averaging over time, 10^{-5} s in case of ^2H NMR experiments.

The linewidth of resonance peaks from oriented samples depends on both the spin–spin relaxation and the residual mosaic spread of bilayer orientation. The influence from mosaic spread is dominant (Arnold et al., 1979). Therefore, smaller mosaic spread results in narrower resonance lines with higher intensity. For oriented bilayers composed of lipids with one perdeuterated hydrocarbon chain, investigated at a field strength of 11.7 Tesla, well resolved spectra with acceptable signal-to-noise ratio can be obtained from 1 mg of membrane material in about 1 h.

2.4.1. Techniques to achieve sample orientation

Lamellar lipid dispersions orient spontaneously at hydrophilic or hydrophobic interfaces. Good substrates for orientation are polished and cleaned

glass surfaces, silicon wafers, and freshly cleaved sheets of mica. If desired, these polar surfaces can be converted into hydrophobic surfaces by chemical reaction with silanes. To achieve orientation, lipids are dissolved in organic solvents like methanol, chloroform, trifluoromethanol, or hexafluoroisopropanol (Bechinger et al., 1996). The solutions are spread over the surface and the solvent is evaporated. Lower lipid concentrations corresponding to fewer lipid layers at the interface and slower drying of an evenly spread solvent layer result in less mosaic spread of bilayer orientation. We obtained better results with adjustment of relative humidity to 80% during evaporation of the organic solvent.

Orientation of bilayers can be also achieved by spreading lipid/water dispersions at interfaces, either as dilute dispersion of small liposomes that is gently dried at the interface in a stream of air with controlled humidity, or as multilamellar dispersion with a water content between 25 and 50 wt.% that is spread and annealed under pressure when sandwiched between two surfaces. Watt's laboratory successfully utilized a method, based on the isopotential spin-dry ultracentrifugation technique (Gröbner et al., 1997). It relies on the centrifugation of membrane fragments onto a solid support with simultaneous, or subsequent, partial evaporation of the solvent to aid alignment.

Reconstituted bilayers containing lipids and membrane proteins like rhodopsin can be oriented at interfaces as well (see Fig. 9). Orientation at interfaces effectively suppresses membrane curvature that in combination with lateral diffusion of lipids averages anisotropic interactions. Furthermore, orientation of membranes at interfaces suppresses magnetic field-induced membrane orientation that disturbs intensity distribution in powder pattern spectra of anisotropic interactions. The field-induced orientation may become severe at the higher field strength of superconductive magnets (Brumm et al., 1992).

2.4.2. Acquisition of spectra with ^2H NMR quadrupole splittings

Two elements are essential to obtain data of high quality: (i) spectra of the lipid matrix that

are acquired with sufficient bandwidth and features that manipulate intensities of the first data points in the FID, like digital filtering, turned off, and (ii) echo sequences like the quadrupolar echo (Davis et al., 1976) that result in spectra without baseline distortions and free of first order phase errors. The stationary solids probe of the spectrometer should be equipped with a flat coil probe that accommodates the oriented sample (Bechinger and Opella, 1991). A probe design that allows changing sample orientation in the magnetic field is desirable (Doty Scientific, Inc.).

The ^2H carrier frequency of the instrument is placed at the center of the resonance spectrum. Deuterium NMR spectra are observed using a quadrupolar echo pulse sequence ($90_x^\circ\text{-}\tau\text{-}90_y^\circ\text{-}\tau\text{-acquire}$) (Davis et al., 1976). The purpose of using an echo sequence is to delay acquisition of the ^2H NMR resonance signal past the decay of electronic ringing of the resonance coil and the preamplifier circuits after the strong radio-frequency

pulses. This ensures registration of resonance signals without distortions of the baseline. The first 90° pulse brings magnetization into the $x\text{-}y$ plane of the rotating coordinate system. Magnetization spreads rapidly under the influence of the strong interaction between the electric quadrupolar moment of the ^2H nucleus and the electric field gradients in $\text{C-}^2\text{H}$ -bonds. The second, phase-shifted 90° pulse refocuses the quadrupole interaction after the delay time τ . The acquisition of the free induction decay begins well in advance of the formation of the quadrupolar echo. The data are acquired at a digitizer dwell time of $2.5\text{ }\mu\text{s}$ or less to ensure sufficient spectral width. Since most ^2H NMR signals have spin-lattice relaxation times of less than 100 ms, the delay time between scans can be as short as 0.25 s. The $\pi/2$ pulses should have a duration of less than $5\text{ }\mu\text{s}$ to ensure homogeneous excitation over the full bandwidth of resonances. The delay time, τ , must be long enough to avoid any perturbation of the

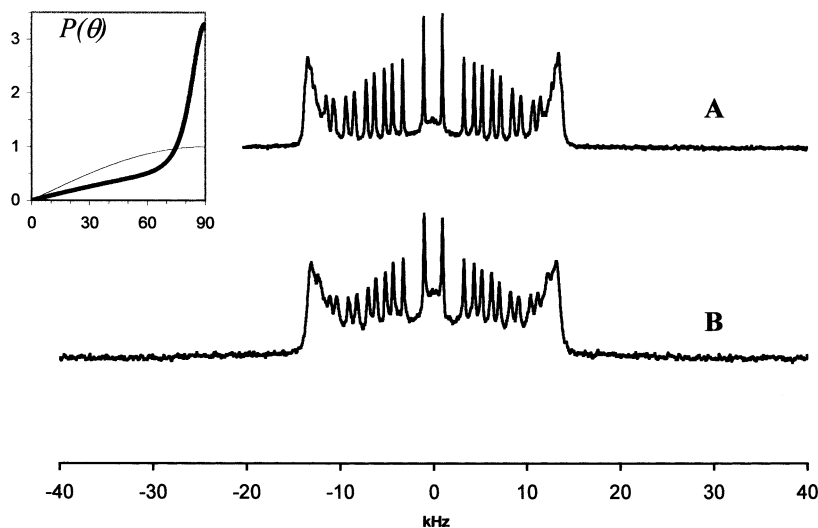


Fig. 9. ^2H NMR spectra of oriented bilayers of 1-stearoyl(d_{35})-2-docosahexaenoyl-sn-glycero-3-phosphocholine ($18:0_{\text{d}_{35}}\text{-}22:6\text{n}3$ PC) without (A) and with (B) reconstituted rhodopsin (molar ratio lipid:rhodopsin = 100:1). Reconstitution was conducted as described in (Litman, 1982). The ^2H NMR signals are from the perdeuterated stearic acid hydrocarbon chain. The insert shows the angular distribution function $p(\theta)$ of bilayer normals of spectrum B (thick line; see (Sternin et al., 2001)). The glass plates with the bilayers are oriented perpendicular to the magnetic field ($\theta = 90^\circ$). For comparison the distribution function of an unoriented sample, $p(\theta) = \sin\theta$, is also shown (thin line). In oriented bilayers with low mosaic spread the ^2H NMR spectra of ^2H nuclei in $\text{C-}^2\text{H}$ -bonds are doublets. The magnitude of the doublet splitting is proportional to the order parameter of the $\text{C-}^2\text{H}$ -bond. The insertion of rhodopsin into the bilayers resulted in a small decrease of chain order. The reduction in order was largest in the region of carbon atoms 8–12 of the saturated hydrocarbon chain.

NMR signal from electronic ringing of the resonance circuits of the probe and the receiver after the ^2H NMR transmitter pulse, typically 50–100 μs .

High-quality processing of the ^2H NMR spectra requires special computer programs (see e.g. Sternin et al., 1983, 2001) that are not provided in commercial NMR software. The time of the echo maximum must be determined with a resolution of one tenth of a dwell time unit by fitting a spline function to the acquired data points. Spline interpolation is also used to calculate free induction decay signals in real and imaginary channels that are time-base corrected to start data processing exactly at the echo maximum. If desired, the spectral width can be reduced by a procedure of digital filtering that was described in a paper by Prosser et al. (Prosser et al., 1991). The processing requires graphical tools to improve adjustment of processing parameters like the spectral phase and the location of the echo maximum. The graphical tools also enable detection of problems with spectrometer settings, e.g. the adjustment of the *rf*-phases and of amplification in the two quadrature detection channels, digitizer instability, etc. We have included these tools into a user-friendly computer program written in MATHCAD (MathSoft, Inc., Cambridge, MA) (Holte et al., 1995).

2.4.3. Extraction of orientational distribution functions and order parameters

Precise knowledge of orientational distribution functions of lipid particles in the magnetic field is desirable for data analysis. The measured spectrum, $I(\omega)$, contains not only information about the effective strength of anisotropic interactions, determined by conformation and motions of lipid molecules in these patches and represented by an anisotropy distribution function, $g(x)$, but also the angular distribution function $p(\theta)$ of bilayer normals (mosaic spread) that describes orientation of lipid aggregates with respect to the magnetic field (Schäfer et al., 1998)

$$I(\omega) = \int p(\theta) \left[g(x) \frac{\partial x}{\partial \omega} \right] d\theta, \quad x = x(\theta, \omega).$$

The extraction of $g(x)$ from spectra with a random distribution of orientations, $p(\theta) = \sin\theta$ by a pro-

cedure called dePakeing has been widely used in lipid research for the past 15 years (Bloom et al., 1981; Sternin et al., 1983). The calculations yield virtual spectra of oriented bilayers without mosaic spread that have their bilayer normal oriented parallel to the magnetic field. The novel numerical approaches using the Tikhonov regularization technique have made it possible to extract not only $g(x)$ but also $p(\theta)$ (Schäfer et al., 1998). We have recently been able to adapt these methods to systems such as lipid bilayers oriented on glass plates (Sternin et al., 2001).

Data analysis of oriented samples, in particular those that have been oriented on glass slides, shows that quite often orientation is less than perfect. When lipid bilayers are oriented with the bilayer normal perpendicular to the magnetic field, it takes surprisingly little preference in orientation to create the false impression that these systems are very well oriented. The use of broad band solid state NMR instruments and appropriate numerical methods reveals the true $p(\theta)$ and $g(x)$ distribution functions (Sternin et al., 2001). Analysis of the spectral distribution function $g(x)$ enables determination of the ^2H NMR order parameters. With the $p(\theta)$ -function the orientation of lipid particles and of membrane-incorporated peptides and proteins are determined. The angular distribution function is also important for interpretation of data from X-ray-diffraction, neutron diffraction, and infrared spectroscopy.

3. Conclusions

Thanks to the use of magic angle spinning techniques and the widespread availability of specific labeling, nearly 100 membrane parameters can be measured, probing every segment of the lipid matrix with spatial resolution at the level of 1 Å and temporal resolution of motions from pico- to ms. The only labels that may be required for NMR studies are non-perturbing substitutions with isotopes that have distinct NMR characteristics, like ^2H and ^{13}C . While part of the NMR information is available for immediate interpretation without the use of computer simulations, a much deeper understanding of lipid structure and dynamics is

achieved when results are compared with the outcome of molecular dynamics simulations of a suitable model membrane. This is particularly useful for the interpretation of relaxation- and cross-relaxation parameters.

The goal of membrane structural studies by NMR is to obtain a precise description of lipid-protein interaction that modulates membrane protein function. The study of lipid-protein interactions requires sensitive, non-perturbing experimental tools. Let us estimate the magnitude of shifts of lipid order parameters related to changes in free energy of a lipid domain containing one membrane protein. Experiments on the metaI–metaII equilibrium of rhodopsin have shown that shifts in the free energy of a lipid-protein complex as small as 0.1 kT (1 kT = 600 cal/mol) are of physiological relevance (Mitchell et al., 1998). Let us assume that the change in free energy is related to an elastic compression of lipid area. A change in free energy of 0.1 kT = 60 cal/mol of a lipid domain containing 60 lipid molecules corresponds to a change in lipid area per molecule of 0.8 \AA^2 , equivalent to a change in average chain order parameters of $\Delta S = \pm 0.008$ (Koenig et al., 1997). The ^2H NMR measurements resolve changes in lipid order parameters of $\Delta S = \pm 0.002$ corresponding to a change in bilayer thickness of $\pm 0.1 \text{ \AA}$ and a change in area per lipid of $\pm 0.2 \text{ \AA}^2$. The NMR methods meet the stringent criteria for detecting such small elastic deformations of the lipid matrix.

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References

- Arnold, K., Gawrisch, K., Volke, F., 1979. ^{31}P NMR investigations of phospholipids I. Dipolar interactions and the ^{31}P NMR lineshape of oriented phospholipid/water dispersions. *Studia Biophysica* 75, 189–197.
- Bechinger, B., Opella, S.J., 1991. Flat-coil probe for NMR spectroscopy of oriented membrane samples. *J. Magn. Reson.* 95, 585–588.
- Bechinger, B., Gierasch, L.M., Montal, M., Zasloff, M., Opella, S.J., 1996. Orientations of helical peptides in membrane bilayers by solid state NMR spectroscopy. *Solid State Nucl. Magn. Reson.* 7, 185–191.
- Bloom, M., Evans, E., 1991. Observation of surface undulations on the mesoscopic length scale by NMR. In: Peliti, L. (Ed.), *Biologically Inspired Physics*. Plenum Press, New York, pp. 137–147.
- Bloom, M., Davis, J.H., MacKay, A.L., 1981. Direct determination of the oriented sample spectrum from the powder spectrum for systems with local axial symmetry. *Chem. Phys. Lett.* 80, 198–202.
- Bremer, J., Mendz, G.L., Moore, W.J., 1984. Skewed exchange spectroscopy-two-dimensional method for the measurement of cross relaxation in ^1H NMR spectroscopy. *J. Am. Chem. Soc.* 106, 4691–4696.
- Brumm, T., Möps, A., Dolainsky, C., Brückner, S., Bayerl, T.M., 1992. Macroscopic orientation effects in broadband NMR-spectra of model membranes at high magnetic field strength—a method preventing such effects. *Biophys. J.* 61, 1018–1024.
- Brüschweiler, R., Wright, P.E., 1994. Water self-diffusion model for protein–water NMR cross-relaxation. *Chem. Phys. Lett.* 229, 75–81.
- Burnett, L.J., Muller, B.H., 1971. *J. Chem. Phys.* 55, 5829–5831.
- Chen, Z.J., Stark, R.E., 1996. Evaluating spin diffusion in MAS-NOESY spectra of phospholipid multibilayers. *Solid State Nucl. Magn. Reson.* 7, 239–246.
- Chen, Z.J., VanGorkom, L.C.M., Epand, R.M., Stark, R.E., 1996. Nuclear magnetic resonance studies of lipid hydration in monomethyl dioleoylphosphatidylethanolamine dispersions. *Biophys. J.* 70, 1412–1418.
- Davis, J.H., Jeffrey, K.R., Bloom, M., Valic, M.I., Higgs, T.P., 1976. Quadrupolar echo deuterium magnetic resonance spectroscopy in ordered hydrocarbon chains. *Chem. Phys. Lett.* 42, 390–394.
- Davis, J.H., Auger, M., Hodges, R.S., 1995. High resolution ^1H nuclear magnetic resonance of a transmembrane peptide. *Biophys. J.* 69, 1917–1932.
- Ellena, J.F., Hutton, W.C., Cafiso, D.S., 1985. Elucidation of cross-relaxation pathways in phospholipid-vesicles utilizing two-dimensional ^1H -NMR spectroscopy. *J. Am. Chem. Soc.* 107, 1530–1537.
- Ernst, R.R., Bodenhausen, G., Wokaun, A., 1991. Dynamic processes studied by two-dimensional exchange spectroscopy. In: *Principles of Nuclear Magnetic Resonance in One and Two Dimensions*. Oxford University Press, Oxford, pp. 490–538.
- Feller, S.E., C.A. Brown, D.T. Nizza, K. Gawrisch, 2002. NMR measurements and MD simulations of ethanol-membrane interactions. *Biophys. J.* 82, 1396–1404.
- Feller, S.E., Huster, D., Gawrisch, K., 1999. Interpretation of NOESY cross-relaxation rates from molecular dynamics

- simulation of a lipid bilayer. *J. Am. Chem. Soc.* 121, 8963–8964.
- Forbes, J., Husted, C., Oldfield, E., 1988a. High-field, high-resolution proton magic-angle sample-spinning nuclear magnetic resonance spectroscopic studies of gel and liquid-crystalline lipid bilayers and the effects of cholesterol. *J. Am. Chem. Soc.* 110, 1059–1065.
- Forbes, J., Bowers, J., Shan, X., Moran, L., Oldfield, E., Moscarello, M.A., 1988b. Some new developments in solid-state nuclear magnetic resonance spectroscopic studies of lipids and biological membranes, including the effects of cholesterol in model and natural systems. *J. Chem. Soc., Faraday Trans.* 184, 3821–3849.
- Fritzshann, T., Hafner, S., Volke, F., Spiess, H.W. MAS-Experimente mit synchron rotierenden Gradienten. 1997. GDCh Diskussionstagung: Magnetische Resonanz und Relaxation: Molekulare und kollektive Dynamik. Conference Proceedings.
- Gabriel, N.E., Roberts, M.F., 1987. Short-chain lecithin long-chain phospholipid unilamellar vesicles-asymmetry, dynamics, and enzymatic-hydrolysis of the short-chain component. *Biochemistry* 26, 2432–2440.
- Gröbner, G., Taylor, A., Williamson, P.T., Choi, G., Glaubitz, C., Watts, J.A., De Grip, W.J., Watts, A., 1997. Macroscopic orientation of natural and model membranes for structural studies. *Anal. Biochem.* 254, 132–138.
- Gross, J.D., Warschawski, D.E., Griffin, R.G., 1997. Dipolar recoupling in MAS NMR: a probe for segmental order in lipid bilayers. *J. Am. Chem. Soc.* 119, 796–802.
- Halladay, H.N., Stark, R.E., Ali, S., Bittman, R., 1990. Magic-angle spinning NMR studies of molecular organization in multibilayers formed by 1-octadecanoyl-2-decanoyl-sn-glycero-3-phosphocholine. *Biophys. J.* 58, 1449–1461.
- Herzfeld, J., Roufosse, A., Haberkorn, R.A., Griffin, R.G., Glimcher, M.J., 1980. Magic angle sample spinning in inhomogeneously broadened biological systems. *Philos. Trans. R Soc. Lond. [Biol.]* 289, 459–469.
- Holte, L.L., Gawrisch, K., 1997. Determining ethanol distribution in phospholipid multilayers with MAS-NOESY spectra. *Biochemistry* 36, 4669–4674.
- Holte, L.L., Peter, S.A., Sinnwell, T.M., Gawrisch, K., 1995. ^2H nuclear magnetic resonance order parameter profiles suggest a change of molecular shape for phosphatidylcholines containing a polyunsaturated acyl chain. *Biophys. J.* 68, 2396–2403.
- Huster, D., Gawrisch, K., 1999. NOESY NMR crosspeaks between lipid headgroups and hydrocarbon chains: spin diffusion or molecular disorder? *J. Am. Chem. Soc.* 121, 1992–1993.
- Huster, D., Arnold, K., Gawrisch, K., 1999. Investigation of lipid organization in biological membranes by two-dimensional nuclear Overhauser enhancement spectroscopy. *J. Phys. Chem. B* 103, 243–251.
- Koenig, B.W., Strey, H.H., Gawrisch, K., 1997. Membrane lateral compressibility determined by NMR and X-ray diffraction: effect of acyl chain polyunsaturation. *Biophys. J.* 73, 1954–1966.
- Lichtenberg, D., Petersen, N.O., Girardet, J., 1975. The interpretation of proton magnetic resonance linewidths for lecithin dispersions: effect of particle size and chain packing. *Biochim. Biophys. Acta* 382, 10–21.
- Litman, B.J., 1982. Purification of rhodopsin by concanavalin-A affinity-chromatography. *Methods Enzymol.* 81, 150–153.
- Macura, S., Huang, Y., Suter, D., Ernst, R.R., 1981. Two-dimensional chemical exchange and cross-relaxation spectroscopy of coupled nuclear spins. *J. Magn. Reson.* 43, 259–281.
- Mehring, P., 1983. Principles of High Resolution NMR in Solids. Springer, Berlin, New York.
- Mitchell, D.C., Gawrisch, K., Litman, B.J., Salem, N., 1998. Why is docosahexaenoic acid essential for nervous system function. *Biochem. Soc. Trans.* 26, 365–370.
- Morrison, C., Bloom, M., 1994. Orientation dependence of ^2H nuclear magnetic resonance spin-lattice relaxation in phospholipid and phospholipid-cholesterol systems. *J. Chem. Phys.* 101, 749–763.
- Nagle, J.F., Liu, Y.F., Tristram-Nagle, S., Epand, R.M., Stark, R.E., 1999. Re-analysis of magic angle spinning nuclear magnetic resonance determination of interlamellar waters in lipid bilayer dispersions. *Biophys. J.* 77, 2062–2065.
- Oldfield, E., Bowers, J.L., Forbes, J., 1987. High-resolution proton and ^{13}C NMR of membranes: why sonicate. *Biochemistry* 26, 6919–6923.
- Pampel, A., Volke, F., 1997. Rotational sidebands in two-dimensional proton high-resolution MAS NMR spectra. *J. Magn. Reson. Anal.* 3, 193–198.
- Perrin, C.L., Gipe, R.K., 1984. Multisite kinetics by quantitative 2-D NMR. *J. Am. Chem. Soc.* 106, 4036–4038.
- Prosser, R.S., Davis, J.H., Dahlquist, F.W., Lindorfer, M.A., 1991. ^2H nuclear magnetic resonance of the gramicidin A backbone in a phospholipid bilayer. *Biochemistry* 30, 4687–4696.
- Schäfer, H., Mädler, B., Sternin, E., 1998. Determination of orientational order parameters from ^2H NMR spectra of magnetically partially oriented lipid bilayers. *Biophys. J.* 74, 1007–1014.
- Seelig, J., 1977. Deuterium magnetic resonance: theory and application to lipid membranes. *Q. Rev. Biophys.* 10, 353–418.
- Seelig, J., 1978. ^{31}P nuclear magnetic resonance and the head group structure of phospholipids in membranes. *Biochim. Biophys. Acta* 515, 105–140.
- Sefcik, M.D., Schaefer, J., Stejskal, E.O., McKay, R.A., Elena, J.F., Dodd, S.W., Brown, M.F., 1983. Lipid bilayer dynamics and rhodopsin-lipid interactions: new approach using high-resolution solid-state ^{13}C NMR. *Biochem. Biophys. Res. Commun.* 114, 1048–1055.
- Simons, K., Ikonen, E., 1997. Functional rafts in cell membranes. *Nature* 387, 569–572.
- Sternin, E., Bloom, M., MacKay, A.L., 1983. De-pake-ing of NMR spectra. *J. Magn. Reson.* 55, 274–282.

- Sternin, E., Schäfer, H., Polozov, I.V., Gawrisch, K., 2001. Simultaneous determination of orientational and order parameter distributions from NMR spectra of partially oriented model membranes. *J. Magn. Reson.* 149, 110–113.
- Tycko, R., Dabbagh, G., Mirau, P.A., 1989. Determination of chemical-shift-anisotropy lineshapes in a two-dimensional magic-angle-spinning NMR experiment. *J. Magn. Reson.* 85, 265–274.
- Volke, F., Pampel, A., 1995. Membrane hydration and structure on a subnanometer scale as seen by high-resolution solid-state nuclear magnetic resonance-POPC and POPC/C12EO4 model membranes. *Biophys. J.* 68, 1960–1965.
- Xu, Z.C., Cafiso, D.S., 1986. Phospholipid packing and conformation in small vesicles revealed by 2-D ^1H nuclear magnetic resonance cross-relaxation spectroscopy. *Biophys. J.* 49, 779–783.
- Yau, W.M., Gawrisch, K., 2000. Lateral lipid diffusion dominates NOESY cross-relaxation in membranes. *J. Am. Chem. Soc.* 122, 3971–3972.
- Yau, W.M., Wimley, W.C., Gawrisch, K., White, S.H., 1998. The preference of tryptophan for membrane interfaces. *Biochemistry* 37, 14713–14718.
- Yeagle, P., 1993. *The Membranes of Cells*. Academic Press, San Diego.
- Yeagle, P.L., Frye, J., 1987. Effects of unsaturation on ^2H -NMR quadrupole splittings and ^{13}C -NMR relaxation in phospholipid bilayers. *Biochim. Biophys. Acta* 899, 137–142.
- Zhou, Z., Sayer, B.G., Stark, R.E., Epand, R.M., 1997. High-resolution magic-angle spinning ^1H nuclear magnetic resonance studies of lipid dispersions using spherical glass ampoules. *Chem. Phys. Lipids* 90, 45–53.
- Zhou, Z., Sayer, B.G., Hughes, D.W., Stark, R.E., Epand, R.M., 1999. Studies of phospholipid hydration by high-resolution magic-angle spinning nuclear magnetic resonance. *Biophys. J.* 76, 387–399.